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Application for Research Grant
(Use extra pages as needed)

Date: July 30, 1974

1. Principal Investigator (give title and degrees):

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Assistant Professor

2. Institution & address:

Department of Physiology
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3. Department(s) where research will be done or collaboration provided:

Department of Physiology

4. Short title of study:

Effect of Nicotine on Respiratory Airway Smooth Muscle and Pregnant Myometrium: Biophysical, Biochemical and Electrophysiological Studies

5. Proposed starting date: January 1, 1975.

6. Estimated time to complete: Three Years

7. Brief description of specific research aims:

Pregnant myometrium (Years 1 & 2)

The proposed research is to be conducted within the context of my ongoing research program on the mechanisms whereby the crucial modification of function of the pregnant uterus is effected at term (i.e. from a relatively quiescent organ to one producing powerful co-ordinated contractions). The specific aim of the proposed research is to investigate mechanisms which might be responsible for the premature delivery of the fetus, a phenomenon which occurs with increased frequency in smoking mothers. In investigating these mechanisms the following possibilities will be considered (see rationale below, section 8): 1. that nicotine affects the production of prostaglandins, the sensitivity of the myometrium to prostaglandins and/or the potentiation by prostaglandins of uterine responses to oxytocin; 2. that nicotine alters the release of acetylcholine in the myometrium; 3. that nicotine affects tissue cyclic AMP content (cyclic AMP has been implicated in mediating inhibition of myometrium); 4. that nicotine affects the electrophysiological properties of the myometrium. Specific experiments utilizing biophysical, biochemical and electrophysiological tools to study the possibilities are detailed below (section 9).

Airway Smooth Muscle (Year 3)

The following studies are proposed within the context of my ongoing research on mechanisms of tone in airway smooth muscle. The specific aims of the proposed research are 1. to document the effects of nicotine on the biophysical and electrophysiological characteristics of canine tracheal smooth muscle (this preparation has the advantage that it is not subject to the uncontrolled and interacting variables present in tracheal ring chains and whole animal preparations on which most previous studies have been conducted); 2. to determine whether all of the effects of nicotine can be attributed to neural mechanisms and; 3. to study the effect of nicotine on the biophysical and electrophysiological effects of tetraethylammonium (TEA) on tracheal smooth muscle. Since TEA converts trachealis muscle from a tonic, multi-unit muscle to a spontaneous phasic single-unit muscle exhibiting action potentials and a myogenic response, the mechanisms underlying these changes and their modification by nicotine could have wide physiological significance. (Details of the rationale and experimental protocol follow in sections 8 and 9 below).

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8. Brief statement of working hypothesis:

Pregnant Myometrium

Recent evidence has linked the activity of the pregnant uterus at term to direct and indirect effects of prostaglandins. Vane and Williams (1973) have shown that 1) the spontaneous activity of isolated term-pregnant rat uterus is inhibited by agents which inhibit prostaglandin synthesis 2) this activity is restored by the addition of PGE_2 and $PGF_{2\alpha}$ to the bathing solution, and 3) the release of $PGF_{2\alpha}$ from the uterus is greatly increased on the day of expected parturition. In addition Karim and Sharma (1971) and Gillespie et al (1972) have adduced evidence for an effect of prostaglandins on oxytocin release from the posterior pituitary in humans and Gillespie (1972) has shown that $PGF_{2\alpha}$ and PGE_2 potentiate the action of oxytocin. Thus, in addressing research to the well documented problem of premature parturition in smoking mothers (Herriot et al, 1962; Underwood et al, 1965; Mulcahy, 1968; Murphy & Mulcahy, 1971) the relationship of the pregnant uterus to prostaglandins and oxytocin would appear to be a key controlling factor of interest. Specifically, the hypothesis to be tested is that nicotine alters the relation of the uterus to prostaglandins and oxytocin so as to produce premature parturition. This might be accomplished by 1) an increased synthesis of prostaglandins 2) an increased sensivity of the myometrium to prostaglandins and/or oxytocin 3) decreasing the effect of prostaglandins on cyclic AMP production, since cyclic AMP has been implicated in inhibition of the myometrium. An effect of nicotine on the release of acetylcholine will also be investigated.

Another factor to be considered in the future pending the outcome of these experiments is the effect

(Continued Appendix 1)

9. Details of experimental design and procedures (append extra pages as necessary)

I Experimental Design

A. Pregnant Myometrium

Since the development of coordinated, strong contractions in term-pregnant myometrium has been related to 1) an increase in the release of prostaglandins E_2 and $F_{2\alpha}$ (Karim, 1968) 2) an increase in the ratio of $PGF_{2\alpha}$: E_2 in labor (Karim, 1968; Liggins, 1973) 3) a potentiation of the effect of oxytocin by $PGF_{2\alpha}$ and E_2 (Gillespie, 1972) as well as 4) extra-uterine effects involving a stimulation of oxytocin release from the posterior pituitary by $PGF_{2\alpha}$ and E_2 (Gillespie et al, 1972) and an effect of these prostaglandins in producing progesterone withdrawal (de Paiva and Csapo, 1973), an evaluation of the mechanism of premature parturition must take into consideration these various influences. The present study focuses on the effect of nicotine on the relationship of prostaglandins and oxytocin to term pregnant uterus. In order to study these effects in a comprehensive way the present proposal involves a multidisciplinary approach utilizing biophysical, biochemical and electrophysiological techniques, all of which are in routine use in my laboratory. The following specific experiments are proposed:

Biophysical

1) Measurement of the effect of nicotine on the basal tone and spontaneous activity of isolated, term pregnant rat myometrium in the presence and absence of atropine and inhibitors (indomethacin, meclofenamate) of prostaglandin synthesis. These experiments are designed to determine the presence of a cholinergic component in the response and to document in detail the mechanical response of our preparation to these agents. This is of particular importance since our recent studies (Marshall & Knoeger, 1973) have shown that the basal tone maintained by the uterus between spontaneous contractions is largely supported by active mechanisms and our as yet unpublished observations show that this basal tone changes qualitatively with the progress of pregnancy. While its relation to the control of uterine function is not yet clear it may be an index of subtle changes effected in the myometrium with advancing pregnancy and should be documented more thoroughly in parallel with observations on the effect of nicotine on the magnitude of contraction.

2) Measurement of the effect of nicotine on the dose-response relation to oxytocin and prostaglandins E_2 and $F_{2\alpha}$ and the potentiation of the effect of oxytocin by the prostaglandins. These experiments should provide a powerful tool in the analysis of the effect of nicotine on both the direct effects of these uterine stimulants as well as their interactive effects.

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APPENDIX 1

8. Of nicotine on progesterone-withdrawal caused by prostaglandins (de Paiva and Csapo, 1973)

Airway Smooth Muscle

The realizations that the calibre of airways is actively regulated by smooth muscle and that this regulation has usually been studied in preparations subject to a variety of uncontrolled and interacting factors (e.g. whole animal preparations, tracheal ring chain Hawkins & Paton, 1958; Widdocombe, 1966) has stimulated my interest in this smooth muscle. We (Stephens et al, 1969; Stephens and Kroeger, 1970; Kroeger and Stephens, 1971) have chosen the canine trachealis muscle preparation as an eminently suitable model for respiratory airway smooth muscle. My recent studies have shown that tetraethylammonium (TEA) produces phasic electrical and mechanical activity in isolated tracheal smooth muscle which is normally quiescent and produces only tonic responses to stimulating agonists. Since both TEA and nicotine have been shown to affect cholinergic neural elements in other preparations, it would be of interest to study the effect of nicotine on this musale directly as well as its influence on TEA-induced responses. Thus the hypothesis to be tested is that the biophysical and electrophysiological effects of nicotine on airway smooth muscle can be attributed to neural mechanisms. The use of TEA as a tool in producing phasic electrical activity should be an invaluable tool in unmasking effects of nicotine on the muscle membrane.

APPENDIX 2

9. Biochemical

1. Measurement of the effect of nicotine on the release of specific prostaglandins. These experiments are designed to test the hypothesis that nicotine alters the synthesis of prostaglandins in the uterus and that the various prostaglandins are affected differentially. In order to obtain some further insight as to the relative importance the decidua and the myometrium in these effects, these structures will be studied independently.

2. Measurement of the effect of nicotine on tissue cyclic AMP levels and on the response of cyclic AMP levels to β-adrenoceptor activation (by isoproterenol). Since a large number of studies have suggested an inhibitory influence of tissue cyclic AMP (Kroeger and Marshall, 1974; Marshall and Kroeger, 1973; Anderson 1973, Triner et al, 1972) and tissue levels as cyclic AMP are very sensitive to stimulatory agonists, being decreased very rapidly (Kroeger & Moorhouse, 1974) these studies should provide a very sensitive index of effects of nicotine on the adenyl cyclase-phosphodiesterase system in whole tissue.

Electrophysiological

1. Measurement of the effect of nicotine on the resting membrane potential and on the height of action potential.

2. Measurement of the effect of nicotine on the cable properties (membrane resistance, length constant) myometrium.

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APPENDIX 2 CONT' D

The measurement the various electrophysiological parameters mentioned above (which, in practise, can all be measured in the same preparation using a Tomita bath, see Procedures below) should permit an analysis of electrophysiological mechanisms of the biophysical effects observed. This data yields evidence concerning membrane permeability, changes in Na and K gradients and changes in conduction velocity, all of which might be involved in mediating the biophysical response.

B. Airway Smooth Muscle

(The following is a brief overview of experiments planned for the latter part, year 3, of the proposed research)

Isolated canine tracheal smooth muscle is of the multi-unit variety (Kroeger and Stephens, in press; Stephens et al, 1969) in that it 1) exhibits no spontaneous mechanical or electrophysiological activity 2) responds to stimulation with graded depolarization and tonic responses and 3) its responses to electrical field stimulation are mediated via intrinsic neural elements. However, our recent finding that in the presence of tetraethylammonium (TEA) the trachealis takes on the properties of single-unit smooth muscle ("spontaneous" phasic electrical and mechanical activity, myogenic response to stretch) suggests that the function of tracheal muscle can be modified quite simply. Hence any understanding of the effect of nicotine on respiratory airways must take into account its effect on both the multi-and single-unit characteristics of this smooth muscle, this phenomenon having been previously overlooked. Previous studies on the effect of nicotine on airway smooth muscle have been carried out in the tracheal ring chain preparation (Hawkins & Paton, 1958) which permits only a gross evaluation of smooth muscle performance since 1) the muscle is subjected to the elastic stress of the cartilaginous ring and 2) the oxygenation of the muscle is impaired due to the presence of the mucosa on one side. While these studies did show neural components to the responses seen, they were not designed to discern effects of nicotine on airway smooth muscle itself. Since our recent evidence suggests that the effect of TEA is on the muscle membrane it seems possible that another ganglionic agent, nicotine may also act on the muscle directly. Thus, in view of this new data, it would be of interest to study the effect of nicotine on the single-unit as well as the multi-unit characteristics of tracheal smooth muscle.

The following experiments are proposed in order to document the effect of nicotine on the multi- and single-unit (with TEA) properties of the trachealis and to differentiate between its actions on neural elements and the muscle per se

Biophysical

1. Measurement of the effect of nicotine on basal and carbachol-induced tone in the trachealis in the presence and absence of atropine and hexamethonium.

2. Measurement of the effect of nicotine on the stimulus-response (electrical field stimulation) relation in the presence and absence of atropine and hexamethonium.

3. Measurement of the effect of nicotine on the mechanical response to TEA, including the dose-response relationship, in the presence and absence of atropine and hexamethonium.

The first two of the above experiments are designed to detect an effect of nicotine on the resting and active properties of this muscle in its multi-unit mode and to define or unmask any neural component of this effect. The third experiment is designed to document the effect of nicotine in the single-unit muscle and to define any cholinergic component in the response.

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APPENDIX 2 CONT'D

Electrophysiological

1. Measurement of the effect of nicotine on the resting membrane potential in the presence and absence of atropine, hexamethonium and TEA.

2. Measurement of the effect of nicotine on the cable properties, membrane resistance and rectification of tracheal muscle in the presence and absence of atropine, hexamethonium and TEA.

3. Measurement of the effect of nicotine on action potentials stimulated in the presence of TEA.

The above experiments are designed to provide a thorough documentation of the effect of nicotine on the electrophysiological properties which could modify the function of single-and multi-unit trachealis smooth muscle.

II Methods of Procedure

A Biophysical Studies

1. Dissection

(a) Myometrium

Term-pregnant Sprague-Dawley rats are used within one day of expected parturition and strips of myometrium obtained according to methods which we have published previously (Kroeger & Marshall, 1973, enclosed). Briefly, the rats are anesthetized with diethyl ether, the uterus removed and the fetuses gently expelled. Longitudinal segments of the anti-mesometrial edge of the uterus are then prepared and placed immediately for equilibration in a Krebs-Ringer solution. The composition of this solution is as follows (in mM): NaCl, 118.5; $NaHCO_3$, 23.8; KCl, 4.7; KH_2PO_4, 2.4; $MgCl_2$, 1.0; $CaCl_2$, 2.5; dextrose 5.5; this is bubbled with a gas mixture containing 95% O_2 and 5% CO_2 so as to obtain a pH of 7.38 at 37°C.

The advantages of this preparation are that 1) it is stable in its biophysical and electrophysiological properties for a number of hours after pre-equilibration, and 2) it contains a good longitudinal muscle layer and a minimum of endometrium.

(b) Trachealis Smooth Muscle

Mongrel dogs, 7-10 Kg in weight are anesthetized with 30 mg/kg sodium pentobarbital I.V. The cervical trachea is then removed and placed immediately in a Krebs-Ringer solution (as above). The dissection is carried out as previously described (Stephens et al, 1969). Briefly, a single ring of the trachea is cut free, the cartilage bisected and the section everted. The mucosa is then easily separated from the muscle layer and removed. The muscle is then tied at its insertions to the cartilage, cut free and placed for pre-equilibration in a Krebs-Ringer solution at 37° C.

The advantages of this preparation are 1) it is very stable in its mechanical and electrophysiological properties for a period of hours after pre-equilibration 2) it contains less than 25% connective and non-muscle tissue 3) muscle fibres are all oriented in the same general direction (parallel-fibred) 4) it can be tetanized and 5) its length-tension diagram shows a clearly defined optimal length at a low resting tension (Stephens et al, 1969).

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APPENDIX 2 CONT'D

2. Recording of Isometric Myograms (from uterus and trachealis)

The lower end of the muscle is tied with 3-0 braided surgical silk to a hook at the bottom of a bath. The upper end is attached to a force transducer (Statham) which is connected to a bridge amplifier and dynograph recorder (Brush, Mark 280). The bath, which is maintained at 37 ± 0.5° C and is constantly bubbled with a 95 - 5% O_2 - CO_2 mixture can be rapidly drained and refilled with fresh solution to remove drugs after responses are complete. The force transducer is mounted on a rack and pinion so as to adjust the length of the muscle as desired. Field stimulation is effected through platinum plate electrodes from a 60Hz source.

3. Measurement of dose-response relationships

All studies are conducted at the optimal length of the muscle. This is determined by stimulating the muscle with a supramaximal field stimulus at various lengths and the length-tension diagram plotted. Force is normalized as grams/cm^2 and the length at which the muscle develops the greatest active tension (resting tension subtracted from total tension) is considered to be the optimal length.

The effect of nicotine on the dose-response relation of myometrium to oxytocin and prostaglandins will be determined as follows: The dose-response relation of the myometrium to oxytocin and prostaglandins will first be determined by adding increasing amounts of these agents to the bath in separate exposure intervals and monitoring the myogram for a standard (1 minute) interval. The bath will be washed after each test and the muscle allowed to re-equilibrate 5 minutes before the next dose is tested. This protocol should prevent desensitization and provide a stable muscle preparation. Since the responses to these agents will not be tonically sustained but will include increases in "spontaneous" phasic activity, the response will be quantified in terms of the area under the myogram (as measured by planimetry). Potentiation of oxytocin responses (cf. Pickles et.al., 1966) will be measured by the simultaneous addition of subthreshold doses of prostaglandins and oxytocin and measuring the contractile response. The effect of nicotine on these various parameters will be assesed by repeating these measurements with the muscle exposed to nicotine (40 - 80 µg/ml).

Measurement of the effect of nicotine on the dose-response relation of trachealis to TEA will also involve planimetry of phasic myograms; however, because desensitization of trachealis to TEA is not observed, cumulative dose-response relations will be measured. In other respects the experimental protocol for these studies is similar to that described above.

4. Stimulus - Response relation

Since responses to electrical stimulation are 95% inhibited by atropine (10^{-7}M), an effect of nicotine on neural elements should be evident in a shift of the stimulus-response relation. These effects will be measured by applying various intensities of electrical fields to muscles pre equilibrated at optimal length. Since the trachealis responds tonically to electrical stimulation the maximum isometric tension will be taken as the response and plotted as a function of the stimulus intensity. These studies will be performed in the presence and absence of atropine and nicotine.

B. Biochemical Studies

1. Measurement of prostaglandin release

The methods used will be essentially those developed by Vane and Williams (1973)

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APPENDIX 2 CONT'D

and Gilmore et.al. (1968) for the extraction and assay of prostaglandins and Green and Samuelson (1964) for the separation of these compounds by thin-layer chromatograph.

After exposure of the bathing solution to the muscle (or decidua) in the presence and absence of nicotine, the solution is collected and the prostaglandins extracted. The latter is accomplished by acidifriction of the solution to pH3 with IN HCl and extraction twice with one volume ethyl acetate. The ethyl acetate phases are pooled and evaporated to dryness at 40°C in the presence of a vacuum. The residue is stored at -12°C until required for assay.

Bioassay for prostaglandin-like activity is done using superfused strips of rat stomach and rat colon in the presence of a variety of antagonists to block responses to other agents which may be present in the extract. Specifically, responses to acetylcholine, 5-hydroxytryptamine, histamine and catecholamines are blocked by the addition of hyosine hydrobromide (10^{-7} g/ml) methysergide maleate (2×10^{-7} g/ml) mepyramine maleate (10^{-7} g/ml) phenoxybenzamine hydrochoride (10^{-7} g/ml) and propranolol hydrochloride (10^{-6} g/ml). Since the rat stomach is about ten times as sensitive to PGE_2 than to $PGF_{2\alpha}$ whereas the rat colon is equally sensitive to the two agents bioassay is accomplished by exposure of these preparations to standard amounts of these prostaglandins and to the muscle extracts. The results of the initial bioassay are confirmed by separating the prostaglandins in the extract by thin-layer chromatography and re-assay. Specifically, a part of the extract is taken up in 0.5 ml absolute ethanol and applied, alongside standard prostaglandins, to glass plates pre-coated with 0.25 mm. silica gel. Plates are developed in the AI solvent system of Green and Samuelson (1964) which contains benzene, dioxane and acetic acid in the ratio 20:20:1. When the solvent front nears the top, the plates are dried at room temperature and the substances visualized by spraying with water. The areas corresponding to the authentic prostaglandins (as well as intermediate areas) are then scraped off into separate tubes, taken up in Krebs-Ringer solution, filtered, and assayed as described previously. Recovery is estimated by including tritium-labelled prostaglandin standards.

2. Measurement of tissue cyclic AMP content

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(a) Muscle equilibration

Strips of term pregnant myometrium are mounted isometrically on glass rods and pre-equilibrated in normal Krebs-Ringer solution as described above. The effect of nicotine on cAMP levels and the response to β-adrenoceptor activation (by isoproterenol, 10^{-6}M) will be measured by equilibrating paired muscle in the presence and absence of nicotine and in in the presence of isoproterenol and isoproterenol + nicotine (40 - 80 µg/ml). At the appropriate time (i.e. at the time of initial and maximal physiological response) muscles are rapidly withdrawn and plunged into liquid N_2. Another protocol which has provided a very sensitive index of changes in the breakdown of cAMP in whole tissue is one in which the tissue cAMP content is first increased by prior exposure to isoproterenol and then the decrease in tissue cAMP monitored during the interval immediately following in which isoproterenol is withdrawn and α- and β-adrenoceptor blocking agents introduced. Since diverse agents which cause contraction have been shown to increase the breakdown of cAMP (Kroeger and Moorhouse, 1974) the effect of nicotine (40 - 80 µg/ml) will be tested in the proposed studies. Specifically, the effect of nicotine will be compared with untreated controls and other

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APPENDIX 2 CONT'D

paired experimental muscle exposed to nicotine and hexamethonium, atropine, propranolol or phentolamine, the latter being designed to differentiate between direct effects on the muscle and nerve-mediated effects.

(b) Biochemical analysis

Frozen tissues are pulverized in the presence of 0.5 M $HClO_4$, extracted at 37° for 4 minutes and centrifuged (2000 g x 2 min.). A triethamolamine - KCl buffer, pH 7.5 is added to the supernatant, the mixture neutralized (pH = 7.5) and centrifuged (2000 g x 4 min.). Aliquots of the supernatant are transferred to small vials and lyophilized. These are then assayed for cAMP by a protein binding assay (obtained in kit form from Amersham-Searle). In this method the experimental samples and "standards" are equilibrated with ^3H cAMP and a cAMP binding protein in the presence of a buffer. At the end of this equilibration (90 min.) activated charcoal is added to all tubes, (to remove unbound ^3H cAMP) the mixture is thoroughly mixed (Vortex, 10 sec) and centrifuged (2000 g x 4 min.). An aliquot of the supernatant is transferred to a scintillation cocktail and counted for ^3H. Thus the number of counts obtained is inversely related to the non-radioactive cAMP present in the tube. The standard curve obtained (after subtraction of appropriate blanks) is linear. This method has proved to be very sensitive, reproducible and free from artefacts in our hands.

C. Electrophysiological Studies

1. Measurement of Membrane potential

The segments are mounted in a 10-ml organ bath maintained at 37 C and arranged for the recording of membrane potentials and tension. Glass microelectrodes of 30-80 MΩ resistance filled with 2 m KCl are used to penetrate the smooth muscle cells. Membrane potentials and tension were displayed on a dual-beam oscilloscope and photographed on 35 mm film. The rate of flow of solution through the organ bath is about 5 ml/min.

The general procedure is to equilibrate the segments in normal solution for 1-2 hr. During equilibration the resting tension (residual tension between spontaneous contractions) is gradually adjusted to about 0.2 g by means of a micromanipulator to which the strain gauge is attached. After equilibration is complete measurements are made of resting membrane potential (maximum level of membrane polarization between spontaneous contractions), action potential and frequency during contraction. The electrical records are accepted only if the electrode remains in the same cell before, during, and after one or more spontaneous contractions. Additional criteria for an acceptable electrode penetration are: 1) an abrupt shift from the extracellular reference potential to a negative resting potential when the electrode enters the cell, 2) a stable resting potential of the same magnitude before and after a spontaneous contraction, 3) action potentials of uniform height and of magnitude equal to or greater than the absolute value of the resting potential, 4) no change in reference potential, electrode resistance, or tip potential after withdrawal of the electrode from the cell.

While the electrode remains within the same cell from which the control measurements are made, the test substance (nicotine) is added to the bathing medium and the resultant effects on membrane potential and contraction are measured. Only those experiments in which the microelectrode remains in the same cell during control and test periods are taken for analysis.

The term membrane potential refers to the potential measured by an intracellular electrode

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APPENDIX 2 CONT'D

relative to the extracellular fluid during a quiescent period between spontaneous contractions and hence the resting membrane potential has a negative sign.

2. Measurement of Electrotonic Potentials

The muscle strip is placed in a muscle chamber similar to that described by Abe and Tomita (1968). In this chamber polarizing current pulses are applied to the tissue in the direction of the long axis of the muscle fibers by means of two silver-silver chloride plate electrodes 1 cm apart. One of the stimulating electrodes serves as a partition and divides the chamber into two compartments, one for stimulating and one for recording. The muscle is passed through a small hole in the partition electrode so that about one-third of the tissue is in the stimulating bath and the remainder in the recording bath. The surface of the electrode facing the recording chamber is coated with araldite. Both chambers are irrigated independently with physiological salt solution at 37 C and gassed with 95% O_2-5% CO_2.

The magnitude of the applied voltage gradient is recorded with silver-silver chloride electrodes, 5 mm apart, placed in the middle of the stimulating bath. The current passing through the tissue is assumed to be directly proportional to the applied voltage gradient. Rectangular pulses (600 msec duration, variable intensity) are delivered to the plate electrodes from a Grass S-4 stimulator. Stimuli are isolated from ground and passed through a load resistor (39 KΩ) to obtain a constant current.

Electrotonic potentials are elicited by electrical stimulation of the muscle during quiescent periods between contractions and are recorded with intracellular microelectrodes in cells located about 1 mm from the partition electrode. Only those experiments in which the electrode remains in the same cell, and hence at the same distance from the partition electrode before and during the action of the test agents are accepted for analysis.

Since the decay of electrotonic potentials is inversely related to membrane resistance these measurements yield evidence concerning the effect of the test drug on membrane permeability (Kroeger, and Marshall, 1973). Also, by determining the current (applied)-voltage (electrotonic potential) relationship at different measured distances from the stimulating electrode the effect of nicotine on the length constant can be determined. By using the stimulating electrodes to elicit action potentials and monitoring these at various measured distances the effect of nicotine on conduction velocity can also be measured. Rectification, a measure of the increase in membrane permeability to K^+ with depolarization as evidenced by a flattening of the current-voltage relation with positive electrotonic potentials will also be investigated as a possible site of action of nicotine on the muscle membranes. Thus these techniques yield a variety of information and permit a thorough analysis of the effect of nicotine on the electrophysiological parameters of the muscle cell membrane.

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D. Estimate of time required for these studies

Since all of the techniques and methods proposed (with the exception of prostaglandin assays) are in routine use in my laboratory, (see reprints attached) there should be a minimum of time required for commencing these studies. (The prostaglandin assays require only slight modifications of procedures already in use here and I do not anticipate major delays in performing this aspect of the study.) On the basis of my past experience with studies of this kind, the following represents what I consider a realistic budget of the time required for the various aspects of this study:

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Year 1: (Myometrium) - All biophysical studies on myometrium
- All cyclic AMP assays
- Prostaglandin assays set up and standardized

Year 2: (Myometrium) - Prostaglandin assays completed
- Electrophysiological studies

Year 3: (Trachealis) - All studies proposed to be completed

The above estimate is based on the assumption that funds for a full-time technician and student are approved. Since these studies involve a variety of interrelated investigative techniques on an important physiological problem this would appear to be a good project on which to train a graduate student.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

My laboratory comprises about 600 square feet of normal laboratory space plus a shielded room for electrophysiological studies which I share with Dr. N. L. Stephens. My present facilities include apparatus for measuring and recording the biophysical properties of muscle (constant temperature organ baths, strain guages, amplifiers and recorder), biochemical apparatus for measuring cyclic AMP(shaker bath, freeze dryer, fluorimeter) and electrophysiological equipment (including a Tomita bath, oscilloscope, 35 mm. camera, electrometer, stimulator and associated calibrating equipment). In addition I have access to preparative and ultracentrifuges, scintillation counters, a cold room, glassware washer, electronics shop and computer terminal which are shared departmental facilities. Thus, while some equipment will have to be purchased in order to accommodate the additional work load of the proposed studies, the space and major facilities are adequate for this work.

11. Additional facilities required:

Since my present apparatus for biophysical studies is used to capacity in ongoing research, the extensive biophysical studies proposed will require the construction of another such apparatus with a capacity for four muscles (to allow paired control and experimental muscles i.e. one control for three experimental muscles as the protocols require). The equipment needed is as follows: one multi-channel recorder, four organ baths, force transducers and coupler-amplifiers and a circulating water bath.

My present equipment and facilities should be adequate for all other aspects of the proposed studies.

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12. Biographical sketches of investigator(s) and other professional personnel (append): (See Appendix 3)

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available):
(See Appendix 4)

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R: REDACTED MATERIAL

APPENDIX 3

12. PERSONAL DATA

Name: KROEGER, Edwin A.

Date of Birth: REDACTED

Marital Status:

Citizenship: REDACTED

Education:

Wheaton College	B.Sc.	1966	Biology
University of Manitoba	M.Sc.	1968	Physiology
University of Manitoba	Ph.D.	1970	Physiology

Positions Held:

1972 -	Scholar of the MRC, Assistant Professor, Department of Physiology, Faculty of of Medicine, University of Manitoba
1971 - 72	Visiting Instructor, Division of Bio-Med Sciences, Brown University
1970 - 71	Research Associate, Division of Bio-Med Science, Brown University
1967 - 70	Demonstrator, Department of Physiology, Faculty of Medicine, University of Manitoba

Participation in Research

1966-1968, Department of Physiology, University of Manitoba

Studies on effect of hypoxia on mechanical properties and electrophysiology of tracheal smooth muscle (under the supervision of Dr. N.L. Stephens). These studies were done in partial fulfillment of the requirements for the M.Sc. degree. Although the title of the project was suggested, its development, execution and adaptation of techniques were my responsibility. In particular, the development of electrophysiological techniques for the study of this muscle required considerable effort since the trachealis has a considerable amount of connective tissue, small diameter muscle fibers, and proved to be electrically quiescent. These characteristics made it difficult not only to penetrate smooth muscle cells but also to ascertain that the electrode was in a cell at a given moment, since pressure artifacts can often mimic cell impalements in causing a change in recorded potential. In the preliminary investigations therefore, to validate the recording characteristics of the instrumentation, I showed that 1) the normal electrical activity of the well-documented smooth muscle preparations could be recorded, and 2) on impalement of trachealis cells, a depolarization could be recorded on increasing the extracellular K^+ concentration.

1968-1970, Department of Physiology, University of Manitoba

Studies on effect of hypoxia on energy and calcium metabolism in tracheal smooth muscle (under the supervision of Dr. N.L. Stephens, in partial fulfillment of the requirements for the Ph.D. degree). In attempting to gain a deeper insight into the mechanisms causing the mechanical effects of hypoxia, it was apparent that this would require a careful definition of cellular energy and calcium metabolism. Since such studies had not been undertaken previously in that laboratory this

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APPENDIX 3 CONT'D

Participation in Research Cont'd.

required the setting up of techniques for the measurement of glycogen, ATP, ADP, AMP, creatine phosphate, Ca^{45} efflux and tissue Ca. In addition our requirement that these parameters be measured in muscles on which we had detailed mechanical information confirming the effect of hypoxia necessitated the modification of these procedures for the small muscles used in mechanical studies. The development and execution of these measurements was my responsibility.

1970-1972, Division of Biological and Medical Science, Brown University, Providence, R.I.

Studies on the mechanism of hyperpolarization and relaxation of uterine smooth muscle in response to beta-adrenergic agents (as a Research Associate of Dr. J.M. Marshall and, Visiting Instructor of Biological and Medical Sciences). Electrophysiological techniques are well established in this laboratory. In the measurement of cyclic AMP, I was fortunate in being allowed the use of Dr. John Fain's facilities. This did, however, require modifications so that the relatively small amounts of this substance found in smooth muscle could be measured. In the measurement of tissue Ca the utility of the relatively new "La-technique" was demonstrated and exploited for demonstrating the small but significant changes in tissue Ca which may be responsible for relaxation. The development and execution of these measurements in this laboratory were my responsibility. These findingswere pursued in collaboration with Dr. C. Paul Bianchi who allowed me the use of his laboratory for the "pulse-labelling" studies. His tutelage on the interpretation of ion flux measurements was invaluable.

1972-1974, Department of Physiology,

During these past years, as a Scholar of the MRC and Assistant Professor of Physiology, I have set up a laboratory in which biophysical, electrophysiological and biochemical studies are currently in progress and have conducted the research on mechanisms of tone in smooth muscle. This has included a documentation of 1) the length-dependency of basal tone in myometrium and its modification by various ionic conditions, 2) the influence of TEA on tone, myogenic response and the electrophysiological properties of tracheal smooth muscle, 3) the cyclic AMP content of myometrium in various ionic and hormonal conditions and 4) the oxygen consumption of myometrium and the effect of various agents which modify tone.

Publications:

Abstracts:

1. Stephens, N.L., E. Kroeger and A. Naimark (1968). Force-velocity relationships of canine tracheal smooth muscle: Effect of hypoxia. Fed. Proc. 27 (2): 228.

2. Stephens, N.L., E. Kroeger and U. Kromer. Properties of airway smooth muscle (Trachealis) mitochondria. P0940 of Abstracts of presentations at the 12th Annual Meeting of the Canadian Thoracic Society, June 1970.

3. Marshall, J.M. and E. Kroeger. Beta-adrenergic effects on rat uterus. 1. Ionic mechanisms of hyperpolarization. The Pharmacologist 13: 315, 1971.

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APPENDIX 3 CONT'D

Abstracts Cont'd

4. Kroeger, E. and J.M. Marshall. Beta-adrenergic effects on rat uterus. 2. Relation to cAMP and Ca. The Pharmacologist 13: 315, 1971.

5. Kroeger, E., N.L. Stephens and R.M. Cherniack. Effect of hypoxia on airway smooth muscle mechanics and electrophysiology. Fed. Proc. 28 (2): 525, 1969.

6. Kroeger, E., N.L. Stephens and R.M. Cherniack. Effect of hypoxia on calcium content and exchange kinetics in airway smooth muscle (ASM). Fed. Proc. 29 (2): 1262, 1970.

7. Kroeger, E., J. Marshall and C. Biancki. Effects of Muscle relaxants on tissue Ca in the rat myometrium. Fed. Proc. 32: 767, 1973.

8. Kroeger, E.A. and J.A. Moorhouse. Initiation of phasic mechanical and electrical activity in tonic tracheal airway smooth muscles (TSM) with tetraethylammonium (TEA^+). Fed. Proc. 32: 389, 1973.

Papers

1. Stephens, N.L., E. Kroeger and J.A. Mehta. Force-velocity characteristics of respiratory airway smooth muscle. J. Appl. Physiol. 26 (6): 685-692, 1969.

2. Stephens, N.L. and E. Kroeger. Effect of hypoxia on airway smooth muscle mechanics and electro-physiology. J. Appl. Physiol. 28 (5): 630-635, 1970.

3. Kroeger, E. and N.L. Stephens. Effect of hypoxia on energy and calcium metabolism in airway smooth muscle. Amer. J. Physiol. 220 (5): 1199-1204, 1971.

4. Stephens, N.L., E.A. Kroeger and U. Kromer. Induction of myogenic response in tonic airway smooth muscle by tetraethylammonium. Amer. J. Physiol. (In Press)

5. Kroeger, E.A. and N.L. Stephens. Effect of tetraethlammonium on tonic airway smooth muscle: Initiation of phasic electrical activity. Amer. J. Physiol. (In Press)

6. Kroeger, E.A. and J.M. Marshall. Beta-adrenergic effects on rat myometrium: Mechanisms of membrane hyperpolarization. Amer. J. Physiol. 225: 1339-1345.

7. Kroeger, E.A. and J.M. Marshall. Beta-adrenergic effects on rat myometrium: Role of cyclic AMP. Amer. J. Physiol. (In Press)

8. Kroeger, E.A., J.M. Marshall, and C.P. Bianchi. Effect of isoproterenol and D-600 on Ca movements in rat myometrium. Submitted to J. Pharmacol. Exp. Therap.

9. Marshall, J.M. and E.A. Kroeger. Adrenergic influences on uterine smooth muscle. Phil. Trans. Roy. Soc. Lond. B 265: 135-148, 1973.

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APPENDIX 4

3. PUBLICATIONS:

Stephens, N.L., E. Kroeger and J.A. Mehta. Force-velocity characteristics of respiratory airway smooth muscle. J. Appl. Physiol. 26 (6): 685-692, 1969.

Kroeger, E. and N.L. Stephens. Effect of hypoxia on energy and calcium metabolism in airway smooth muscle. Amer. J. Physiol. 220 (5): 1199-1204, 1971.

✓ Kroeger, E.A. and N.L. Stephens. Effect of tetraethlammonium on tonic airway smooth muscle: Initiation of phasic electrical activity. Amer. J. Physiol. (In Press)

Kroeger, E.A. and J.M. Marshall. Beta-adrenergic effects on rat myometrium: Mechanisms of membrane hyperpolarization. Amer. J. Physiol. 225: 1339-1345.

Kroeger, E.A. and J.M. Marshall. Beta-adrenergic effects on rat myometrium: Role of cyclic AMP. Amer. J. Physiol. (In Press).

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R: REDACTED MATERIAL

14. First year budget:

	% time	Amount
A. Salaries (give names or state "to be recruited")		
Professional (give % time of investigator(s) even if no salary requested)		
E. A. Kroeger		REDACTED
Student - to be recruited		
Technical		
One experienced technician - to be recruited		REDACTED
Sub-Total for A		R
B. Consumable supplies (by major categories)		
Animals - timed pregnant rats, 200 ($8.00 each)		1,600
- boarding charges 40 (.03/diem)		428
Chemicals - for physiological salt solutions, scintillation counting cocktails, biochemical assays		1,800
Glassware and disposable supplies		200
Gas mixtures (for equilibrating physiological salt solutions)		100
Chart paper (for recorder - 6 rolls ($8.40 each)		50.40
Sub-Total for B		$ 4,178.40
C. Other expenses (itemize)		
Equipment maintenance		200
Glassware washer (1 hour /day at $2.00)		520
Sub-Total for C		720
Running Total of A + B + C		$16,898.40
D. Permanent equipment (itemize).		
Oscillograph recorder (see justification above #11) Brush, Mark 440 - 4 channel		3,350.
Amplifier (4) and transducer couplers (4) Brush ($775.00 each)		3,100
Organ baths (4) ($50.00 each)		200
Circulating water bath		345
Force transducers (4): Statham ($180.00 each)		720
Rack and pinion assemblies (4) ($90.00 each)		360
Sub-Total for D		$ 8,075
E. Indirect costs (16% of A+B+C)		$ 2, 534.76
Total request		$27, 508.16

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	13,000	5,000	1,000	1,700	2,850	23,550
Year 3	14,000	2,500	600	-	2,565	19,665

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The mechanism of production and regulation of tone in mammalian smooth muscle: Biophysical, electrophysiological and biochemical studies	Medical Research Council MA 4765	$ 8,400	April 1, 1974 March 31, 1975.
The effect of cyclic AMP on Ca transport of plasma membrane of pregnant myometrium	Banting Research Foundation	7,200	July 1, 1974 June 30, 1975

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Role of Ca & Mg in the biophysical and electrophysiological response of tracheal smooth muscle to tetraethylammonium	Medical Research Council	15,000	April 1, 1975 March 31, 1976.

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Edwin A. Kroeger

Signature Edwin C. Kroeger Date July 29, 1974

Telephone 204 786-3733
Area Code Number Extension

Checks payable to
University of Manitoba

Mailing address for checks
Comptroller's Office, The University of Manitoba
Winnipeg, Manitoba, Canada

Responsible officer of institution

Typed Name R.A. Lebrun

Title Executive Assistant to the President

Signature [illegible] Date JUL 30 1974

Telephone 204 474-8308
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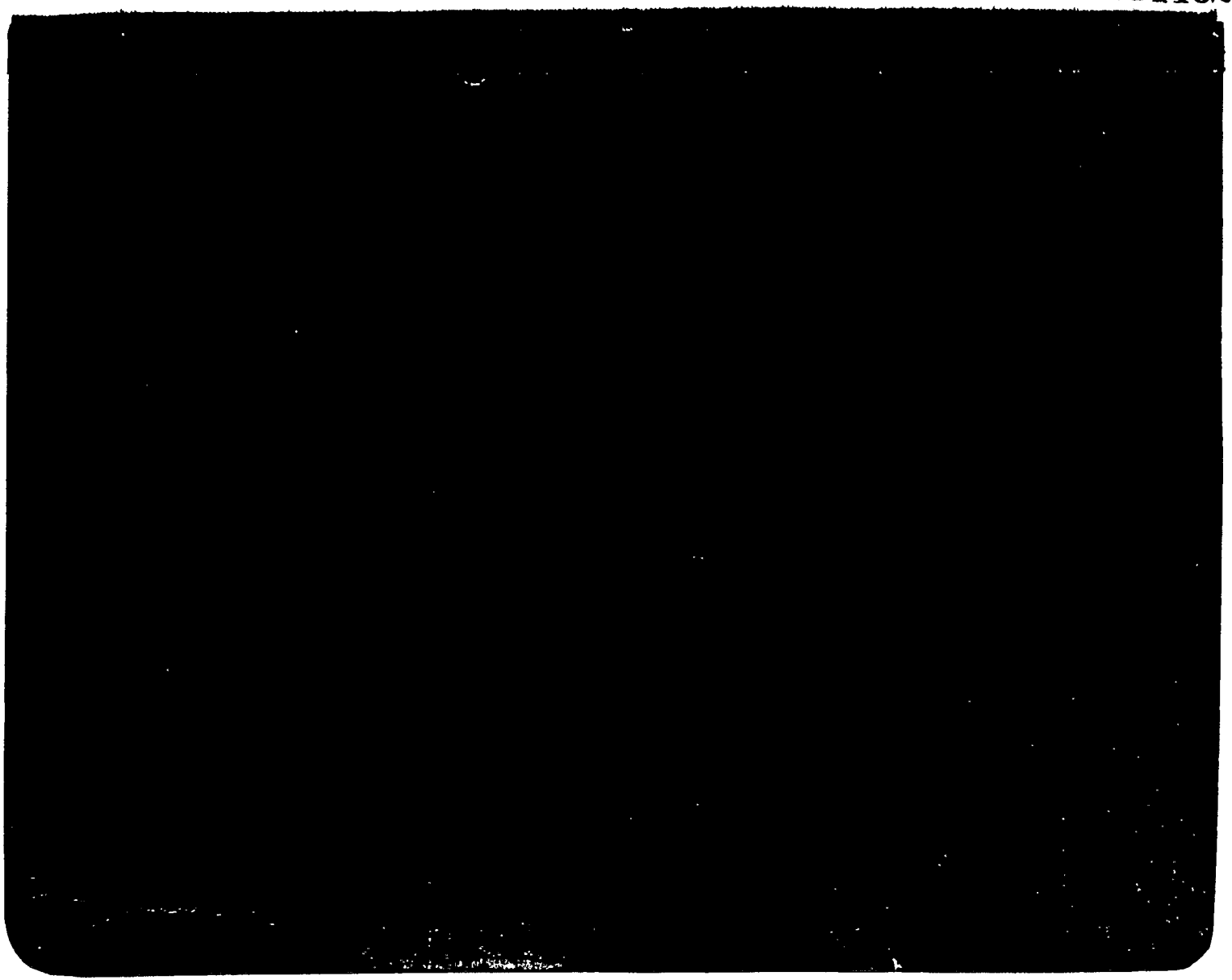

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